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Research Note—

Tissue Tropism and Bursal Transformation Ability of Subgroup J Avian Leukosis Virus in White Leghorn Chickens

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SUMMARY. In Experiment 1, a monoclonal antibody against the envelope glycoprotein (gp85) of subgroup J avian leukosis virus (ALV-J) was used to study the distribution of ALV-J in various tissues of White Leghorn chickens inoculated as embryos with the strain ADOL-Hc1 of ALV-J. At 2 and 6 wk of age, various tissues from infected and control uninfected chickens were tested for the presence of ALV-J gp85 by immunohistochemistry. In Experiment 2, using the methyl green-pyronine (MGP) stain, sections of bursa of Fabricius (BF) from chickens of line $15I_5 \times 7_1$, inoculated with ALV-J or Rous-associated virus-1 (RAV-1), a subgroup A ALV, at hatch were examined for transformation of bursal follicles at 4 and 10 wk of age. In Experiment 1, specific staining indicative of the presence of ALV-J gp85 was noted at both 2 and 6 wk of age in the adrenal gland, bursa, gonads, heart, kidney, liver, bone marrow, nerve, pancreas, proventriculus, spleen, and thymus. In Experiment 2, by 10 wk of age, transformed bursal follicles were detected in MGP-stained sections of BF in only one of five (20%) chickens inoculated with ALV-J at hatch, compared with five of five (100%) chickens inoculated with RAV-1. The data demonstrate distribution of ALV-J gp85 in various tissues of White Leghorn chickens experimentally inoculated as embryos with the virus. The data also confirm our previous observation that ALV-J is capable of inducing transformation of bursal follicles, albeit the incidence is less frequent than that induced by subgroup A ALV.

RESUMEN. *Nota de Investigación*—Tropismo y capacidad de transformación de la bolsa de Fabricio por el subgrupo J del virus de la leucosis aviar en aves blancas tipo Leghorn.

En el experimento 1 se empleó un anticuerpo monoclonal contra la glicoproteína 85 (gp85) del virus del subgrupo J de la leucosis aviar con el fin de estudiar la distribución del virus en varios tejidos de aves blancas tipo Leghorn inoculadas a edad embrionaria con la cepa ADOL-Hc1 del virus J de la leucosis aviar. Se evaluó la presencia de la gp85 mediante la prueba de inmunohistoquímica en varios tejidos de aves infectadas y aves control no infectadas a las 2 y 6 semanas de edad. En el experimento 2, mediante el uso de la tinción de metil verde pironina se examinó la transformación de los folículos de la bolsa de Fabricio a las 4 y 10 semanas de edad en secciones de bolsa de Fabricio de aves de la línea $15I_5 \times 7_1$, inoculadas al nacimiento con el virus de la leucosis aviar subgrupo J o el virus asociado al sarcoma de Rous tipo 1, y el subgrupo A del virus de la leucosis aviar. En el experimento 1, la tinción específica indicó la presencia de la gp85 del virus de la leucosis aviar subgrupo J a las 2 y 6 semanas de edad en la glándula adrenal, bolsa de Fabricio, gónadas, corazón, riñón, hígado, médula ósea, nervio, páncreas, proventrículo, bazo y timo. En el experimento 2, a las 10 semanas de edad, se detectó la transformación de los

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folículos de la bolsa de Fabricio en secciones teñidas con metil verde pironina únicamente en una de las 5 aves (20%) inoculadas al nacimiento con el virus de la leucosis aviar subgrupo J, al ser comparadas con las aves inoculadas con el virus asociado al sarcoma de Rous tipo 1 en las cuales la tinción fue detectada en 5 de las 5 aves (100%). Los datos demuestran la distribución de la gp85 del virus de la leucosis aviar subgrupo J en varios tejidos de aves blancas tipo Leghorn inoculadas experimentalmente a edad embrionaria con el virus. Los datos igualmente confirman nuestra observación previa de la capacidad del virus de la leucosis aviar tipo J de inducir transformación de los folículos de la bolsa de Fabricio aunque su incidencia es menos frecuente a la inducida por el subgrupo A del virus de la leucosis aviar.

Key words: avian leukosis virus subgroup J, tissue tropism, immunohistochemistry, White Leghorn chickens, bursal transformation

Abbreviations: ALV = avian leukosis virus; ALV-J = avian leukosis virus subgroup J; ADOL = Avian Disease and Oncology Laboratory; BF = bursa of Fabricius; CEF = chicken embryo fibroblasts; ELISA = enzyme-linked immunosorbent assay; gp85 = glycoprotein 85; gs = group specific; IU = infectious units; LL = lymphoid leukosis; MGP = methyl green-pyronine; PBS = phosphate buffered saline; RAV-1 = Rous associated virus-1; VI = virus isolation; VN = virus neutralization

Various methods have been used to demonstrate tissue tropism of avian leukosis viruses (ALVs) (6,7,14,22,25,27). These previous reports primarily addressed tissue tropism of ALVs that are known to cause lymphoid leukosis (LL), regardless of virus subgroup. Various tissues exhibited varying degrees of ALV group-specific (gs) antigen, also known as p27 in chickens inoculated with Rous associated virus 1 (RAV-1), a subgroup A ALV; expression of p27 antigen was detected more frequently in bone marrow and bursa of Fabricius (BF) than in spleen and thymus (3). Brown and Robinson (4) found that RAV-1 had high tropism for BF and muscle compared with RAV-2, a subgroup B ALV, which had high tropism for BF and thymus. Robinson *et al.* (22) reported that although RAV-1 proviral DNA can integrate into various tissues, expression of gs and envelope proteins differed in tissues, such as muscles where gs proteins are higher than envelope proteins.

Subgroup J ALV (ALV-J) was first described in the early 1990s in the U.K. (18) and later described in the United States during the late 1990s (11). Myeloid leukosis (ML) is the predominate neoplasm induced by ALV-J, as reported in both field cases and laboratory trials. Natural outbreak with this neoplasm are typically seen in meat-type chickens, although ML was recently diagnosed in commercial layer flocks (17,30). In 1997, Arshad *et al.* (1) evaluated tissue tropism of strain HPRS-103 of ALV-J in meat-type and Brown Leghorn egg-type chickens using immunohistochemistry to test for the presence of ALV-p27; expression of p27 was highest in the adrenal gland, heart, kidney, and proventriculus. Most recently, using the same monoclonal antibody (21) used in the present study, the most intense ALV-J gp85 staining was localized in the adrenal gland, heart, kidney, and proventriculus of naturally infected broilers (13). ALV-J tumor response in White Leghorn chickens is known to be different from that in meat-type chickens (10,18,19,20); however, tissue distribution of ALV-J gp85 has been reported in naturally infected broiler (13), but not White Leghorn, chickens.

The pathogenesis of ALV-induced lymphoid leukosis (LL) involves the formation of preneoplastic bursal follicles; eventually, these preneoplastic follicles develop into bursal lymphoma and metastasize to various organs (10). Experimentally, strain ADOL-Hc1 of ALV-J has been shown to induce a low incidence of LL in various lines of White Leghorn chickens (29), but whether ALV-J can induce a high incidence of transformation of bursal follicles in chickens that are highly susceptible to ALV-induced LL is not known.

The purpose of this study was to evaluate tissue tropism of ALV-J in White Leghorn chickens by immunohistochemistry using a highly specific monoclonal antibody against ALV-J gp85 (21). Using methyl green-pyronine (MGP) stain, we also examined the ability of ALV-J to transform bursal follicles in chickens that are highly susceptible to ALV-induced LL, namely chickens of line $15I_5 \times 7_1$.

MATERIALS AND METHODS

Chickens. Chickens of line $15I_5 \times 7_1$ raised and maintained at the Avian Disease and Oncology Laboratory (ADOL), East Lansing, MI, were used.

Chickens were inoculated with strain ADOL-Hc1 as 7-day-old embryos in Expt. 1 or at hatch in Expt. 2. The breeder flock was free from many avian pathogens, including ALV, as determined by routine serological surveys. Chickens were housed by treatment group in Horsfall isolation units for up to 10 wk and given food and water *ad libitum.*

Viruses. Strain ADOL-Hc1 of ALV-J (11) and strain RAV-1, a subgroup A ALV (10), were used. Viruses were propagated and titrated in C/E (resistant to infection with endogenous ALVs) line 0 chicken embryo fibroblasts (CEFs).

Virological and serological assays. Virus isolation (VI) and virus neutralization (VN) assays were conducted, as previously described (12). Briefly, for VI assays, samples were inoculated on C/E CEFs; 7–9 days later, cell lysates were tested for the presence of the ALV gs (p27) antigen by an enzyme-linked immunosorbent assay (ELISA), as previously described (24). For VN assays, plasma samples were diluted 1:5 with tissue culture media without serum and heat inactivated for 30 min at 56 C. Equal volumes of plasma and virus were incubated for 45 min at 37 C before adding the mixture to C/E CEFs (12); cultures were evaluated for residual virus (presence of p27) by ELISA, as previously described (24).

Immunohistochemistry. Tissue sections were removed from 60% methanol and embedded in paraffin and sectioned at 4 μm. A monoclonal antibody against ALV-J gp85 (21) was used in immunohistochemistry evaluation of various tissues for the presence of ALV-J gp85. Modifications of the procedure used by Arshad *et al.* (1) for staining the tissues was used and included the following with two 2-min washes between each step: A 3% H_2O_2 in methanol solution was applied to the slides for 60 min to block endogenous peroxidases. Super Block, a non–species-specific protein-blocking agent, (Scytek, Logan, UT) was applied to the slides for 5 min, followed by an application of a blocking agent for endogenous avidin/biotin for 5 min. The primary antibody was diluted at 1:200 in normal antibody diluent (Scytek) (from concentrate) and applied by hand to the slide (100μl) and incubated for 60 min. A Vector kit (Vector Labs, Burlingame, CA) using a biotinylated anti-mouse/anti-rabbit (in horse) antibody diluted at 1:136 in normal antibody diluent for 30 min was applied, followed by the application of a Vector Ready-to-Use (R.T.U.) ABC (avidin-biotin complex) for 30 min. Slides were counterstained with Nova Red (Vector Labs), rinsed, dehydrated, cleared, and cover-slipped with a synthetic mounting media. Several negative controls, including a system control that had normal horse sera diluted at 1:28 instead of the primary antibody for 60 min on a section of infected tissues, uninfected tissues treated with primary antibody, and uninfected tissues treated with normal horse sera, were used. Tissues were microscopically examined for the presence of red pigment within the cell cytoplasm, indicating viral antigen expression.

Tissue sections were scored by counting the cells that stained positive; cell types not specific to an organ, such as connective tissue, smooth muscle components in the gastrointestinal tract, and endothelial cells, were not evaluated. Cells in four randomly selected fields of each tissue on a slide were examined and counted at 400×. The average number of cells within the four fields was determined, and a positive-cell staining average was given to the tissue. The overall average score for each tissue was determined by combining the score given for all tissue samples and divided by the respective number of sections examined. The presence of gp85-positive cells specific for a certain tissue were scored as none (no staining), mild (1–25 positive cells), moderate, (26–75 positive cells), and marked (≥76 positive cells).

Transformation of bursal follicles as determined by MGP stain. Sections of bursal plicae stained with MGP were microscopically examined for the presence of transformed bursal follicles. Evidence of transformation included increased pyroninophilia and disruption of normal architecture of bursal follicles as compared with surrounding normal follicles (23,29). If one follicle was positive for transformation, the tissue was considered positive.

Experimental design. *Experiment 1: Tissue tropism.* In this experiment, 30 7-day-old embryos were each inoculated with 10^4 IU (infectious units) of strain ADOL-Hc1 via the yolk sac. Another group of 30 7-day-old embryos served as uninfected controls; each control embryo was inoculated via the yolk sac with 0.1 ml of sterile phosphate buffered saline (PBS). All chicks were sampled for viremia at hatch. At 2 and 6 wk of age, chickens were tested for viremia; also, 10 chickens from each group were randomly selected and euthanatized for collection of adrenal glands, kidney, pancreas, spleen, heart, proventriculus, skeletal muscle, bone marrow, thymus, BF, gonads, and sciatic nerve. Tissues were fixed in 10% neutral-buffered formalin over night and transferred to 60% methanol until processed for immunohistochemistry.

Experiment 2: Transformation of bursal follicles. Fifty day-old chicks were divided into three groups. In groups 1 and 2, 19 chicks were each inoculated intra-abdominally with 10^4 IU of ADOL-Hc1 or with RAV-1, respectively. In group 3, 12 chickens served as uninoculated controls. At 4 and 10 wk of age, chickens were tested for ALV viremia; also, five chickens from each group were euthanatized, and bursal tissues were collected for microscopic evaluation. Transformation of bursal follicles was determined by microscopic examination of bursal tissue stained with MGP (23,29). The plicae were separated and placed flat in cassettes (8–12 plicae using 2–3 cassettes per chicken). Tissues were fixed in 4% paraformaldehyde in PBS, embedded in paraffin, sectioned, and stained with MGP stain (5,23).

Table 1. Distribution of ALV-J gp85 in various tissues of $15I_5 \times 7_1$ chickens inoculated with a virus as 7-day-old embryos.

Tissue	Age at testing (2 wk)	Age at testing (6 wk)
Adrenal gland	7/8 (74)[A]	9/9 (139)
Bursa	6/9 (47)	8/9 (73)
Gonad	7/8 (73)	9/9 (67)
Heart	9/10 (57)	9/9 (83)
Kidney	9/10 (69)	9/9 (121)
Liver	8/9 (46)	9/9 (46)
Bone marrow	5/8 (16)	6/6 (36)
Nerve	7/10 (53)	9/9 (68)
Pancreas	9/10 (62)	9/9 (125)
Proventriculus	10/10 (91)	9/9 (137)
Spleen	8/10 (79)	9/9 (116)
Thymus	9/10 (30)	9/9 (60)

[A]Number of positive chickens/number of examined (average number of tissue specific cells staining positive).

RESULTS AND DISCUSSION

Tissue tropism of ALV-J. In Experiment 1, all chickens inoculated as embryos with ALV-J tested positive for virus at hatch, 2, and 6 wk of age (data not shown), confirming that chickens were infected with the virus as embryos. The distribution of ALV-J gp85 in cells from various organs is summarized in Table 1. Because skeletal muscle from uninoculated control chickens had high background staining that could not be eliminated, data from evaluation of skeletal muscle was not included; no staining was noted in other tissues from control uninoculated chickens (data not shown). At 2 wk of age, proventriculus (glandular epithelial cells), spleen (reticuloendothelial cells and lymphocytes), and adrenal glands (cortical and medullary) had the highest average number of cells stained for ALV-J gp85 expression. At 6 wk of age, adrenal gland (139 cells), proventriculus (137 cells), pancreas (125 acinar and islets of Langerhans cells), kidney (121 distal tubule epithelial cells), and spleen (116 cells) had the highest average number of cells specifically stained for ALV-J gp85. Our data agree with those reported for ALV-J (1,13) and for other subgroups of ALV (7,8,27). In the present study, no mononuclear cell infiltrates were noted in nerve sections examined, and ALV-J gp85 was more frequently detected in sciatic nerve than has been previously reported (1). Previous reports on failure to detect ALV antigen (p27) in nerve tissue (7,8,9) can be explained by differences in expression of p27 and gp85 in different tissues.

At both 2 and 6 wk of age, bone marrow had the least amount of cells staining for the antigen expression with an average of 16 and 36, respectively. Positive staining cell counts used in the present study only demonstrated the ability of viruses to replicate in various tissues, but have limited use in any quantitative analysis because normal cell density varies with tissue and age. Fig. 1 illustrates positive ALV-J gp85 staining in various tissues. In BF, more intense staining of gp85 was observed at the cortical-medullary junction of the follicles, with much less staining of the medulla in some follicles. Expression of ALV-J, as detected by monoclonal antibody to gp85, was observed in most of the bursal sections examined. Previous work by others indicated that when using an antibody against p27, positive staining was mainly detected in the medulla, with some staining in cortex and the surface epithelium (1). Also, in the present study, expression of ALV-J gp85 was detected in the bone marrow in both erythrocyte and leukocyte lineages. In previous reports, the inability to detect gs antigen in the bone marrow of chickens infected with ALV-J was explained by low sensitivity of the antibody (1). Another possible explanation for differences in the ability to detect antigens in bone marrow reported in the present study and in previous reports may also be due to a difference in the expression of gp85 and p27 in various cells and tissues.

Using electron microscopy in previous studies, viral particles were noted in all tissues except the nerve tissue of chickens congenitally infected with strain F42 of subgroup A ALV(8); viral matrix inclusion bodies were noted in myocardium of ALV-infected chickens (15). In attempts to understand the pathogenesis of ALV, an antibody against ALV gs antigen (p27) has been widely used to demonstrate the distribution of ALV in various tissues (1,7,9,14,16,25,26,27). However, the antibody against p27 is not very specific because it may react with all the subgroups of ALV, including endogenous ALV. Other methods used for studying ALV tissue tropism included Southern-blot analysis (3) and *in situ* hybridization (2,28). Only *in situ* hybridization allows for the detection of either DNA or RNA in fixed tissues and, in this case, detects proviral genome or viral transcripts in specific cell types. Using the same ALV-J gp85 monoclonal antibody (21), our results suggest that tissue distribution of ALV-J gp85 in various tissues of White Leghorn chickens is comparable to what has been previously reported for naturally infected broilers (13), regardless of the fact that ALV-J tumor

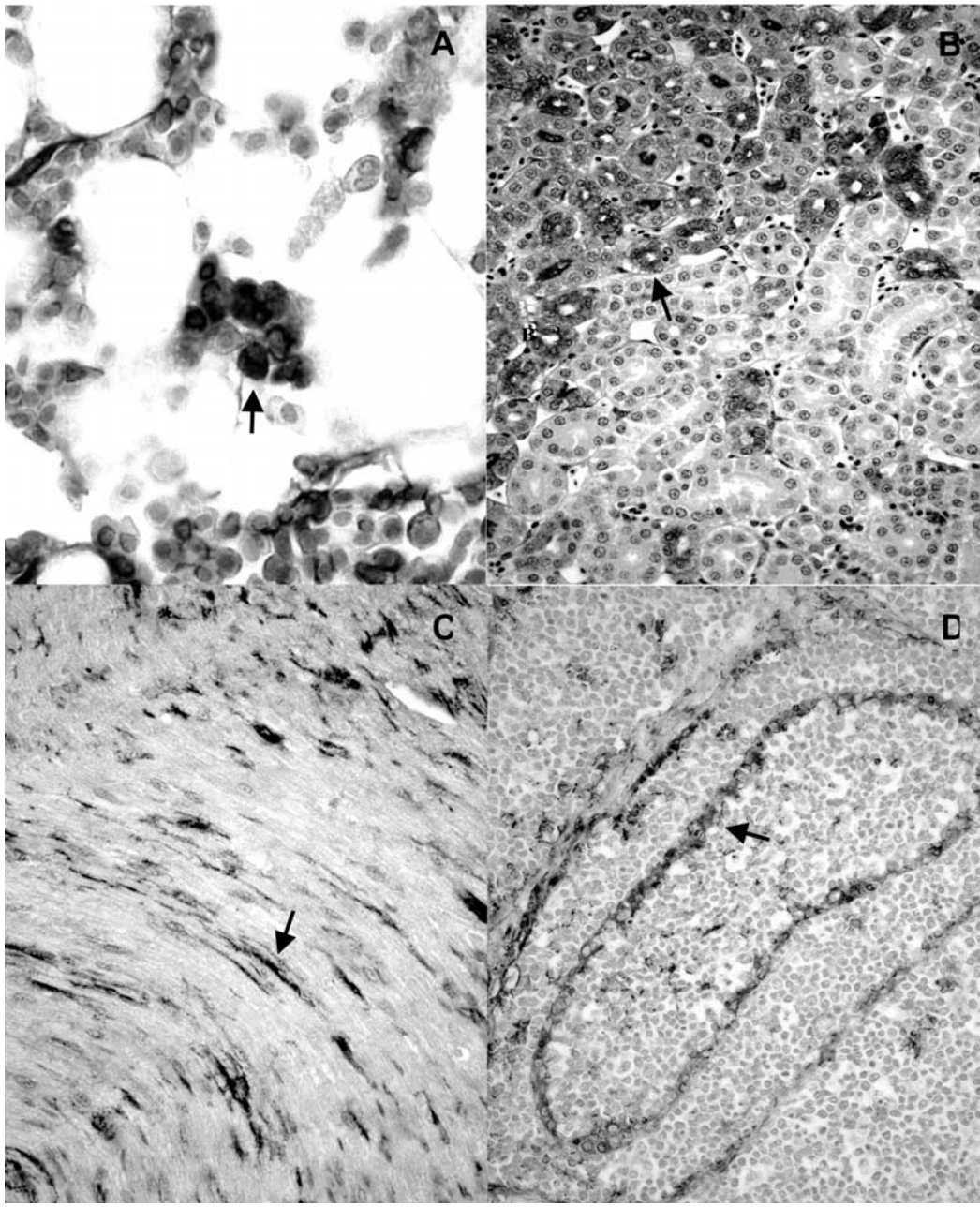

Fig. 1. (A) Bone marrow: various cell lineages demonstrating positive immunohistochemical staining. 1000×. (B) Kidney: distal tubules staining positive. 400×. (C) Sciatic nerve: axonal staining. 400×. (D) Bursa of Fabricius: staining present at intersection of cortex and medulla of bursa follicles.

response in broilers is different from that in White Leghorns (10,18,19,20).

Bursal transformation induced by ALV-J. Typical lesions of transformation of bursal follicles (10) were detected in virus-inoculated groups only (data not shown). At 4 and 10 wk of age, two of five (40%) and five of five (100%) chickens inoculated with RAV-1, a subgroup A ALV, had evidence of

bursal transformation. In contrast, only one of five (20%) chickens inoculated with ALV-J had evidence of bursal transformation at either 4 or 10 wk of age. No lesions were noted in bursa of control uninoculated chickens; also, there was no serological evidence of cross infection between RAV-1, ADOL-Hc1, and control uninoculated groups (data not shown). Not all cell types in bursa that test positive for ALV replication (as demonstrated by immunohistochemistry using monoclonal antibodies) undergo transformation (10). Further, only the presence of typical lesions of transformation of bursal follicles, as detected by MGP-stained sections, are pathogenomonic for LL and is used in early diagnosis of the disease (10). Results from Expt. 2 confirm our previous observation (29) that ALV-J is capable of transforming bursal follicles: however, this is the first time that the ability of strain ADOL-Hc1 of ALV-J to induce LL was tested in chickens of line $15I_5 \times 7_1$, a line that is highly susceptible to ALV-induced LL. Although the number of chickens examined in the present study was relatively small, the data clearly demonstrated differences in ability of ALV-J and ALV-A to induce transformation of bursal follicles in $15I_5 \times 7_1$ chickens. Interestingly, LL has never been recognized as a neoplasm in chickens experimentally inoculated with strain HPRS-103 of ALV–J (19,20). These discrepancies between HPRS-103 and ADOL-Hc1 in their ability to induce LL can be explained by the fact that various strains of ALV may differ in their ability to induce certain tumors (10). However, more studies are required to determine factors that influence development of LL in ALV-J–infected chickens.

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